Highly purified mycobacterial phosphatidylinositol mannosides drive cell-mediated responses and activate NKT cells in cattle

Pirson, Chris; Engel, Regina; Jones, Gareth; Holder, Thomas; Holst, Otto; Vordermeier, H. Martin; Burns, D. L.

DOI:
10.1128/CVI.00638-14

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Pirson, C, Engel, R, Jones, G, Holder, T, Holst, O, Vordermeier, HM & Burns, DL (ed.) 2015, 'Highly purified mycobacterial phosphatidylinositol mannosides drive cell-mediated responses and activate NKT cells in cattle', *Clinical and Vaccine Immunology*, vol. 22, no. 2, pp. 178-184. https://doi.org/10.1128/CVI.00638-14

CVI Journals.ASM.org

Highly Purified Mycobacterial Phosphatidylinositol Mannosides Drive Cell-Mediated Responses and Activate NKT Cells in Cattle

Chris Pirson,[a] Regina Engel,[b] Gareth J. Jones,[a] Thomas Holder,[a] Otto Holst,[b] H. Martin Vordermeier[a]

TB Research Group, Animal Health and Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, United Kingdom[a]; Division of Structural Biochemistry, Research Centre Borstel, Leibniz-Centre for Medicine and Biosciences, Borstel, Germany[b]

Mycobacterial lipids play an important role in the modulation of the immune response upon contact with the host. Using novel methods, we have isolated highly purified phosphatidylinositol mannoside (PIM) molecules (phosphatidylinositol dimannoside [PIM_2], acylphosphatidylinositol dimannoside [$AcPIM_2$], diacyl-phosphatidylinositol dimannoside [Ac_2PIM_2], acylphosphatidylinositol hexamannoside [$AcPIM_6$], and diacylphosphatidylinositol hexamannoside [Ac_2PIM_6]) from virulent *Mycobacterium tuberculosis* to assess their potential to stimulate peripheral blood mononuclear cell (PBMC) responses in *Mycobacterium bovis*-infected cattle. Of these molecules, one ($AcPIM_6$) induced significant levels of gamma interferon (IFN-γ) in bovine PBMCs. Three PIM molecules ($AcPIM_6$, Ac_2PIM_2, and Ac_2PIM_6) were shown to drive significant proliferation in bovine PBMCs. $AcPIM_6$ was subsequently used to phenotype the proliferating cells by flow cytometry. This analysis demonstrated that $AcPIM_6$ was predominantly recognized by $CD3^+$ $CD335^+$ NKT cells. In conclusion, we have identified PIM lipid molecules that interact with bovine lymphocyte populations, and these lipids may be useful as future subunit vaccines or diagnostic reagents. Further, these data demonstrate, for the first time, lipid-specific NKT activation in cattle.

Members of the mycobacterial genus are renowned for their waxy, lipid-rich outer envelope. Under physiological conditions, this outer layer is likely to be the first point of contact between the bacterial cell and the host's immune system, and the outcome of this interaction is pivotal in the establishment of infection. One of the most important groups of membrane bound lipids consists of the phosphatidylinositol mannosides (PIMs). Interest in PIMs was stimulated since it was shown that phosphatidylinositol dimannoside (PIM_2) forms the phosphoglycolipid anchor which tethers a large array of glycolipids and lipoglycans, including lipomannan (LM) and lipoarabinomannan (LAM), to the cellular membrane (1). PIMs have been shown to interact with a variety of immune components and mediate significant effects on the host. Ever since the realization that NKT cells could respond to lipid antigens (2–4) and the subsequent discovery of the CD1d-restricted lipid antigen α-galactosylceramide (α-GalCer) (5, 6), much research effort has been concentrated on understanding lipid antigens. Although work was initially focused on invariant NKT cells, it has since been shown that great diversity exists in the lipid-responsive T cell receptor (TCR) repertoire (7–9) and that these diverse NKT cells contribute to the Th1/Th2 balance (7, 10). It has been shown that CD1d-restricted NKT cells are capable of recognizing a variety of lipid antigens, including phospholipids (11). More recently, a CD1b-restricted subset of T cells has been found (12). Similarly to CD1d-restricted invariant NKT (iNKT) cells, the CD1b-restricted variant cells require CD1B for their development and produce proinflammatory cytokines in response to CD1b-expressing dendritic cells (DCs) (12). It is clear that NKT-like cells have a significant role to play in lipid-mediated responses, and the hunt for their antigens has continued (13–15).

Given the ability of lipid molecules to generate responses in peripheral blood mononuclear cells (PBMC), we decided to assess the ability of individual, highly purified natural PIMs to activate bovine lymphocytes. In this study, we developed a novel extraction method which allowed us to extract and highly purify a variety of PIM molecules from virulent *Mycobacterium tuberculosis* H37Rv. The ability of these molecules to induce lymphocyte responses in *Mycobacterium bovis*-infected cattle was investigated by measuring lymphocyte proliferation and gamma interferon (IFN-γ) production. Furthermore, flow cytometry techniques were utilized to characterize responding cell populations.

MATERIALS AND METHODS

Extraction of PIMs. Using the novel methodology outlined below, highly pure phosphatidylinositol dimannoside (PIM_2), acylphosphatidylinositol dimannoside ($AcPIM_2$), diacyl-phosphatidylinositol dimannoside (Ac_2PIM_2), acylphosphatidylinositol hexamannoside ($AcPIM_6$), and diacylphosphatidylinositol hexamannoside (Ac_2PIM_6) were successfully isolated. Individual PIM molecules were analyzed by electrospray ionization mass spectrometry (ESI-MS) to confirm identity and purity as shown in Fig. 1. Up to 1 g of dry bacterial mass of *Mycobacterium tuberculosis* H37Rv was suspended in 20 to 30 ml of H_2O and ruptured utilizing a French press at a minimum of 20,000 kPa. This procedure was performed five times, and the combined sample was lyophilized.

Up to 0.5 g of this lyophilized material was extracted three times according to the method of Bligh and Dyer (16). The dry mass was suspended in 4 ml of H_2O and washed twice in an additional 2 ml of H_2O

Received 1 October 2014 **Returned for modification** 23 October 2014
Accepted 25 November 2014

Accepted manuscript posted online 10 December 2014

Citation Pirson C, Engel R, Jones GJ, Holder T, Holst O, Vordermeier HM. 2015. Highly purified mycobacterial phosphatidylinositol mannosides drive cell-mediated responses and activate NKT cells in cattle. Clin Vaccine Immunol 22:178–184. doi:10.1128/CVI.00638-14.

Editor: D. L. Burns

Address correspondence to Chris Pirson, chris.pirson@ahvla.gsi.gov.uk.

C.P. and R.E. are joint first authors and contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/CVI.00638-14.

doi:10.1128/CVI.00638-14

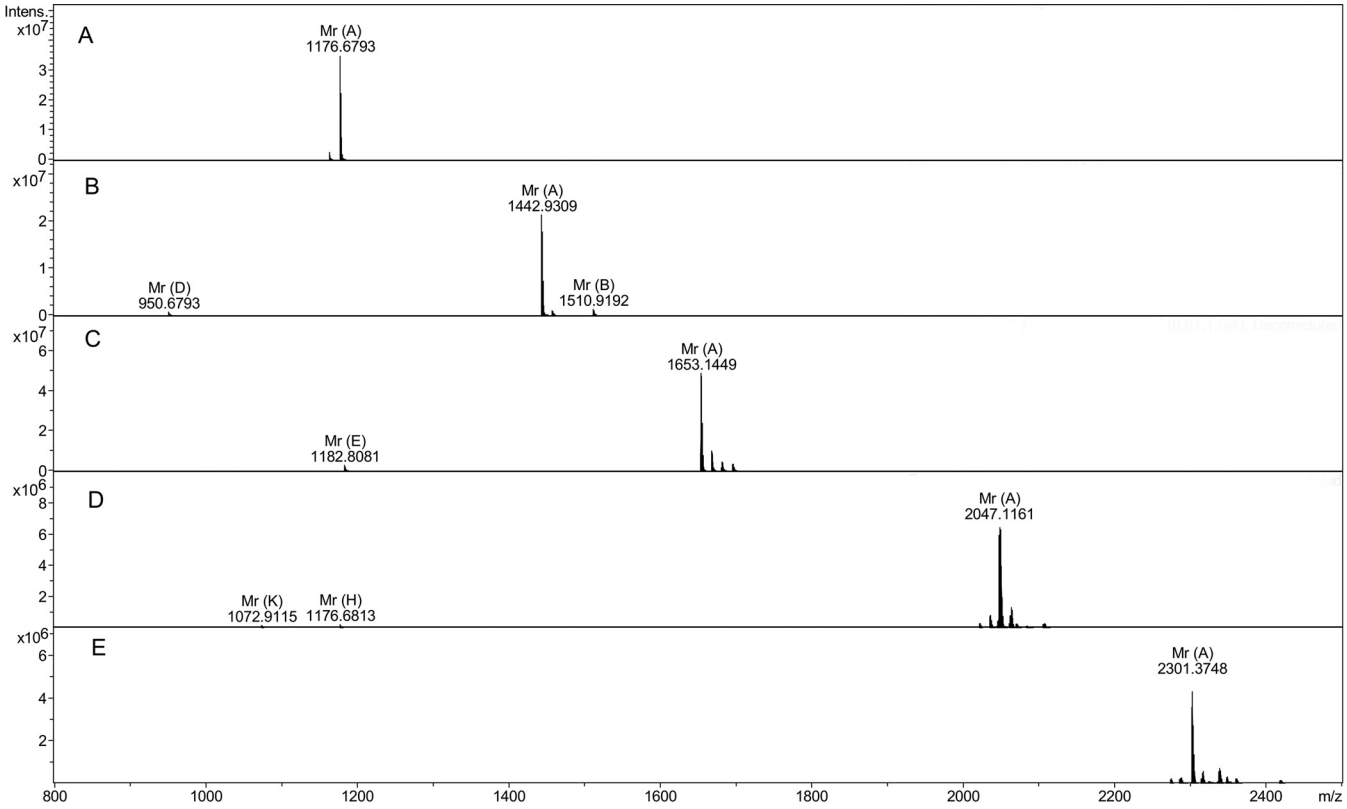

FIG 1 Mass spectrometric proof of purity of isolated PIMs. Identified molecular masses of PIM_2 (calculated molecular mass, 1176.6784 u) (A), $AcPIM_2$ (calculated molecular mass, 1442.9394 u) (B), Ac_2PIM_2 (calculated molecular mass, 1653.1378 u) (C), $AcPIM_6$ (calculated molecular mass, 20147.0881 u) (D), and Ac_2PIM_6 (calculated molecular mass, 2301.3491 u) (E). Letters in parentheses identify the peaks.

before being transferred into a 100-ml Erlenmeyer flask. To this sample was added 30 ml of $CHCl_3$·CH_3OH (1:2, vol/vol), and the sample was sonicated (Branson Sonifier 250; output 2, 40% duty cycle, 20 min). Then, 10 ml of $CHCl_3$ was added, and the sample was sonicated for a further 5 min. Subsequently, an additional 10 ml of H_2O was added, and the sample was sonicated for a final 5 min. The sample was decanted evenly into between two and four 50-ml Nalgene Teflon tubes and centrifuged for 30 min at 10,000 × *g* to generate three phases (a water phase, a $CHCl_3$ phase, and an interphase). The water phases (containing LAM and LM) were removed, combined, and lyophilized, while the $CHCl_3$ phases were transferred into a single 100-ml pear-shaped flask. The total yield of material after three extractions was about 11% of the bacterial dry mass.

Since the remaining interphases contained a lot of $AcPIM_6$, they were suspended in 8 ml of H_2O and combined into 30-ml Kimble high-speed glass tubes before being lyophilized. These phases were then extracted overnight with 30 ml of $CHCl_3$·MeOH (1:2, vol/vol) using a shaker. The sample was then centrifuged for 30 min at 10,000 × *g*, and the $CHCl_3$·MeOH phase was removed. This phase was dried under an N_2 gas stream before being resuspended in equal parts of $CHCl_3$·MeOH, filtered through a 0.2-μm-pore-size polytetrafluoroethylene (PTFE) filter, and dried under N_2. This extraction was repeated two times until no PIMs could be identified (total yield of about 2% of the bacterial dry mass). The obtained PIMs were further purified with silica gel 60 chromatography (see below), as were the $CHCl_3$ phases of the Bligh and Dyer extraction.

Purification of PIMs. All samples were separated on a column (7 by 1 cm) of silica gel 60 (0.04 to 0.063 mm) which was successively eluted with (i) 80 ml of $CHCl_3$·MeOH (8:2, vol/vol), (ii) 60 ml of $CHCl_3$·MeOH (1:1, vol/vol), and finally (iii) 40 ml of $CHCl_3$·MeOH·H_2O (10:10:3, vol/vol/vol). Coextracted cardiolipin and other lipids eluted in the first mobile phase, most PIMs eluted in the second, and the rest of the PIMs eluted in the third. Fractions ii and iii were dried under N_2, resuspended in 10 ml of $CHCl_3$·MeOH (8:2, vol/vol), and passed through a 0.2-μm-pore-size PTFE filter before being analyzed by high-performance thin-layer chromatography (HPTLC). HPTLC was performed using glass-backed 10- by 10-cm silica gel 60 plates (Merck KGAA, Darmstadt, Germany) run in $CHCl_3$·MeOH·H_2O (10:8:2, vol/vol/vol) and stained with Hanessian's stain (0.5 g of ceric sulfate and 25 g of ammonium molybdate in 470 ml of water supplemented with 30 ml of sulfuric acid with stirring) and visualized at 150°C.

Fractions ii and iii from both of the $CHCl_3$ phases of the Bligh and Dyer extraction and the PIMs extracted from the interphase were then further separated by high-performance liquid chromatography (HPLC) using 5-μm Kromasil 100 C_{18} columns (250 by 20 mm) eluted with eluent A ($CHCl_3$·MeOH·H_2O [240:1,140:620, vol/vol/vol] containing 10 mM $NH_4CH_3CO_2$) and eluent B ($CHCl_3$·MeOH [1,400:600, vol/vol] containing 50 mM $NH_4CH_3CO_2$). The initial eluent B gradient was 15% for 60 min, followed by 20% for 140 min, 40% for 80 min, and finally 100% for 60 min at 4 ml min^{-1}. Samples were detected by a light-scattering detector (Sedex; nitrogen pressure, 2×10^5 Pa; temperature, 50°C; split, 1:70). Samples were applied in 200 μl of $CHCl_3$·MeOH (8:2, vol/vol). For analytical runs, 10 μg of sample was injected while 10 mg was injected for preparative separations.

Since PIM_2 and $AcPIM_6$ coeluted on the C_{18} column, they were separated by HPLC on 5-μm Prontosil 200-5-C_{30} reverse-phase columns (250 by 4.6 mm) using the same elution reagents. The initial eluent B gradient was 5% for 5 min, followed by 10% for 15 min, 15% for 50 min, and finally 100% for 10 min at 0.8 ml min^{-1}. Samples were injected as a mixture of 0.6 mg in 80 μl of $CHCl_3$·CH_3OH·H_2O (10:10:3, vol/vol/vol) and detected by the light-scattering detector described above.

Cattle. Blood samples were obtained from 10 naturally infected, single intradermal comparative cervical tuberculin test-positive reactors (between 6 and 36 months of age). Animals were sourced from herds with confirmed bovine tuberculosis breakdowns in Devon, Herefordshire, or Worcestershire and were housed at the Animal Health and Veterinary Laboratories Agency (AHVLA) at the time of blood sampling. Infection was confirmed by necropsy and *M. bovis* culture in all animals. All procedures involving animals were carried out under a project license granted by the Home Office of Great Britain under the Animals (Scientific Procedures) Act 1986. This project was approved by the local VLA Animal Ethics Committee prior to submission to the Home Office.

Isolation of bovine PBMC from whole blood. Whole blood was mixed in equal parts with sterile Hanks balanced salt solution (HBSS) containing 10 U ml^{-1} heparin. This mixture was overlaid onto Histopaque 1077 (Sigma-Aldrich) and centrifuged at 800 × *g* for 40 min. The PBMC interface was removed using a pastette and washed twice in HBSS containing heparin. Live cells were identified via trypan blue exclusion and enumerated using a hemocytometer.

Preparation of lipid antigen suspensions. Briefly, lipids were suspended in an aqueous phase for use in cell culture experiments after removal of $CHCl_3$·CH_3OH by evaporation using an N_2 gas stream. Cell culture medium was added to the dried lipid, and the mixture was subjected to two cycles of heating at 80°C and then sonication for 5 min. Lipids were used to stimulate cells *in vitro* at 20 μg ml^{-1} in all assays.

Lymphocyte proliferation assay. Bovine PBMC were prepared from all 10 animals as described above and were cultured in complete cell culture medium (RPMI 1640 medium containing 25 mM HEPES, 10% fetal calf serum [FCS], 1% nonessential amino acids [NEAA], 5 × 10^{-5} mM β2-mercaptoethanol, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin [Gibco Life Technologies, Paisley, United Kingdom]) at 37°C in 5% CO_2 for 5 days in the presence of antigen at 2 × 10^5 cells $well^{-1}$. After 5 days, cells were pulsed with 1 μCi $well^{-1}$ of [^{3}H]thymidine overnight, before being harvested using a Harvester 96 Mach III (TomTec, Inc., Hamden, CT, USA). Lymphocyte proliferation was assessed by the increased cellular incorporation of [^{3}H]thymidine (cpm), which was measured using a MicroBeta^2 2450 plate counter (PerkinElmer, Waltham, MA, USA). Responses to individual PIMs were considered positive if the cpm exceeded the mean plus 2 times standard deviation of cpm for non-antigen-stimulated cultures from all 10 animals.

Measurement of IFN-γ by Bovigam ELISA. Levels of IFN-γ in 5-day supernatants from the proliferation assay were determined using a Bovigam enzyme-linked immunosorbent assay (ELISA) kit (Prionics AG, Switzerland). Responses to individual PIMs were considered positive if the optical density at 450 nm (OD_{450}) exceeded the mean plus 2 times the standard deviation of the OD_{450} for non-antigen-stimulated cultures from all 10 animals.

Measurement of proliferation and phenotyping by flow cytometry. Bovine PBMC were isolated as described above and labeled with CellTrace violet (Invitrogen Molecular Probes, Paisley, United Kingdom) in accordance with the manufacturer's instructions. Briefly, PBMC were suspended at 1 × 10^7 cells ml^{-1} in prewarmed phosphate-buffered saline (PBS), and 5 mM CellTrace violet was added to a final working concentration of 1 μM. Cells were incubated at 37°C for 20 min before unbound dye was quenched with five times the labeling volume of complete cell culture medium at 37°C for 5 min. Finally, cells were pelleted and washed in prewarmed complete cell culture medium, plated at 2 × 10^5 cells $well^{-1}$, and incubated at 37°C in 5% CO_2 for 5 days in the presence of antigen.

Cultured cells were harvested and resuspended in flow cytometry buffer (PBS containing 2% FCS and 0.05% NaN_3) and labeled for 15 min with the Near-IR live/dead indicator NIRViD (Invitrogen Life Technologies, Paisley, United Kingdom) and mouse anti-bovine CD335, also known as NKp46 (AKS1; AbD Serotec, Oxfordshire, United Kingdom). Cells were washed in flow cytometry buffer, and secondary labeling of anti-CD335 was performed using a 1:400 dilution of rat anti-mouse IgG2a conjugated to allophycocyanin for a further 15 min. After a subsequent wash, cells were further labeled with combinations of *R*-phycoerythrin (*R*-PE)-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine CD3 (MM1A; WSU Monoclonal Antibody Centre, Pullman, Washington, USA), mouse anti-bovine CD4 conjugated to Alexa Fluor 647 (CC30; AbD Serotec, Oxfordshire, United Kingdom), mouse anti-bovine CD8 conjugated to Alexa Fluor 647 (CC63; AbD Serotec, Oxfordshire, United Kingdom), and Alexa Fluor 488-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine γδ-TCR1 (GB21a; WSU Monoclonal Antibody Centre, Pullman, Washington, USA). Finally, labeled cells were washed in flow cytometry buffer and resuspended in 150 μl of 2% paraformaldehyde (Cytofix; BD Biosciences, Oxfordshire, United Kingdom) for at least 30 min at 4°C before analysis on a CyAn ADP analyzer. For capture and analysis, initial gating was on single, NIRViD^{lo} (live) cells into a subsequent lymphocyte gate before gating on CellTrace violet^{lo} cells.

Data and statistical analysis. All data representation and statistical analysis were performed using GraphPad Prism, version 5.04, and GraphPad InStat, version 3.06 (GraphPad Software, La Jolla, CA, USA). Statistical analysis of IFN-γ and lymphocyte proliferation data was performed using a nonparametric repeated-measures analysis of variance (ANOVA; Friedman test) with a Dunn's multiple comparisons posttest.

RESULTS

Highly purified PIM molecules can be isolated from virulent mycobacteria. PIM molecules differing in the number of acyl and mannose residues were highly purified from *M. tuberculosis*. Five distinct PIM molecules were isolated: PIM_2, $AcPIM_2$, Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6. In total, these lipids constituted 3% of the bacterial dry mass after silica gel 60 separation. The ability of the purification method to isolate highly pure PIMs is shown in Fig. 1, in which the structures and purity of the different PIM molecules were confirmed by ESI-MS. In addition, bands corresponding to $AcPIM_2$ molecules (that differed only in the number of carbon atoms in the acyl chains) were clearly resolved by thin-layer chromatography (TLC) analysis (see Fig. S1 in the supplemental material).

Purified PIM molecules activate lymphocytes from *M. bovis*-infected cattle. In order to assess the ability of purified PIMs to induce *in vitro* immune responses in cattle, PBMC from 10 naturally *M. bovis*-infected cattle were cultured for 5 days in the presence of each PIM molecule, and the level of IFN-γ was measured by ELISA (Fig. 2A). Both the frequency and strength of IFN-γ responses differed depending on the nature of the PIM molecule. $AcPIM_2$ was least recognized, inducing responses in only 2 of the 10 animals. PIM_2, Ac_2PIM_2, and Ac_2PIM_6 were more frequently recognized, with responses detected in 3 (PIM_2) and 4 (Ac_2PIM_2 and Ac_2PIM_6) out of 10 animals. $AcPIM_6$ was most frequently recognized, inducing responses in half of the animals studied. Furthermore, $AcPIM_6$ was the only PIM molecule to induce significantly greater levels ($P < 0.01$) of IFN-γ overall than nonstimulated controls.

In addition to measuring IFN-γ production, we also investigated the ability of the PIM molecules to induce PBMC proliferative responses in the same animals. Again, the frequency of responding animals differed depending upon the nature of the PIM molecule (Fig. 2B). PIM_2 failed to induce a proliferative response in any of the animals studied, while $AcPIM_2$ induced responses in only 3 out of 10 animals. In contrast, Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6 were more frequently recognized, inducing proliferative responses in 6 (Ac_2PIM_2 and $AcPIM_6$) and 7 (Ac_2PIM_6) out of 10 animals. Overall, significantly greater PBMC proliferation was de-

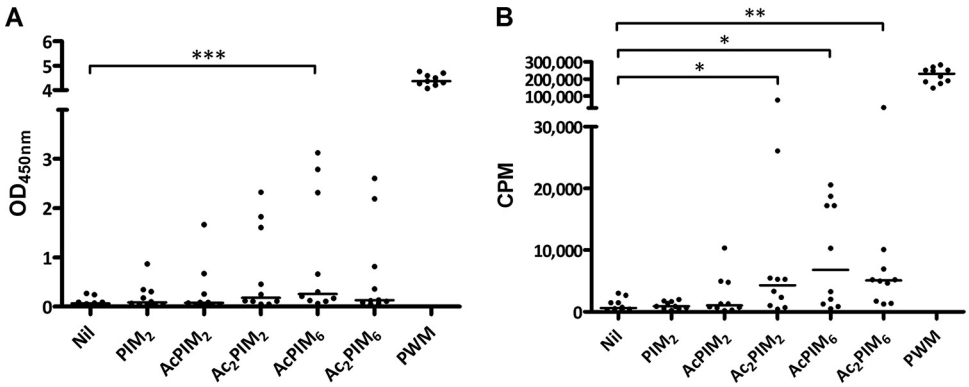

FIG 2 (A) PIM-driven IFN-γ production as measured by Bovigam ELISA. PBMC were incubated with PIMs at 20 μg ml^{-1} for 5 days. Points represent mean responses from triplicate wells for each of the 10 animals tested; lines represent the sample median. ***, $P < 0.001$. (B) PIM-driven proliferation of bovine PBMC as measured by [^{3}H]Thy incorporation. Bovine PBMC were stimulated for 5 days with individual PIMs at 20 μg ml^{-1}. Points represent mean responses from triplicate wells for each of the 10 animals tested; lines represent the sample median. *, $P < 0.05$; **, $P < 0.01$. Nil, nonstimulated controls.

tected in the Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6 treatment groups than in nonstimulated controls, with median values tending to be greater following $AcPIM_6$ stimulation.

Phenotyping of $AcPIM_6$-responsive proliferating cells by flow cytometry. As $AcPIM_6$ was the only PIM molecule to generate significantly increased levels of IFN-γ (Fig. 2A) and produced the greatest increase in the median proliferative response (Fig. 2B), we used this antigen to stimulate PBMC from an *M. bovis*-infected animal to characterize the proliferating cell populations by flow cytometry. Purified protein derivative from *M. bovis* (PPD-B) was used as a control antigen. CellTrace violet-labeled cells were incubated for 5 days with antigen before being harvested and labeled for flow cytometric analysis. After stimulation with either PPD-B or $AcPIM_6$, three populations of proliferating cells were identified based on cell surface phenotyping: (i) $CD4^+$ T cells ($CD3^+$ $CD4^+$), (ii) $CD8^+$ T cells ($CD3^+$ $CD8^+$), and (iii) NKT-like cells ($CD3^+$ $CD335^+$). An example of the gating strategy for identifying proliferating NKT cells is highlighted in Fig. 3A, which demonstrates a greater level of proliferating cells in response to stimulation with $AcPIM_6$ (58.29%) than in the non-antigen-stimulated control (29.87%).

The effects of stimulation with either PPD-B or $AcPIM_6$ on the three different cell populations are summarized in Fig. 3B. Stimulation with PPD-B drove antigen-specific proliferation of approximately 60% of the $CD4^+$ T cells ($CD3^+$ $CD4^+$). Similarly, an antigen-specific proliferative response was seen in approximately 15% of the $CD8^+$ T cells ($CD3^+$ $CD8^+$) to PPD-B. A slight increase in NKT cell ($CD3^+$ $CD335^+$) proliferative responses (approximately 20%) was also seen to these antigens (Fig. 3B).

Stimulation with $AcPIM_6$ induced only limited proliferation of $CD4^+$ T cells (approximately 5%) and no proliferation of $CD8^+$ T cells above the background (Fig. 3B). In contrast, approximately 30% of the NKT cell population mounted a proliferative response after stimulation with $AcPIM_6$ (Fig. 3B). Little or no proliferation above the unstimulated negative control was seen in the $CD3^-$ $CD335^+$ cells or in the $CD3^+$ γδ-TCR^+ populations (data not shown).

DISCUSSION

Mycobacterial lipids have long been implicated in the induction of responses in both the innate and adaptive cell-mediated immune responses (17–21). Although one strategy has been published which allows the isolation of PIMs from the avirulent *M. bovis* BCG strain (18, 22–24) and *M. tuberculosis* H37Rv (22) and certain synthetic molecules (17, 19, 21), in this study we successfully developed a novel method for extracting and subsequently highly purifying and characterizing individual PIM species from the polar fraction of virulent *M. tuberculosis* H37Rv. The method developed here improves upon the previously published protocols primarily by using a French press to disrupt the bacterial cells, thereby increasing PIM yield. Other refinements include the removal of the hot acetone incubation and the use of different reverse-phase conditions for PIM purification. Our strategy allowed us to isolate a greater yield of more highly purified PIMs, as confirmed by ESI-MS, that could be subsequently assayed for their ability to generate responses in lymphocytes.

To assess the ability of these highly purified PIMs to drive immune responses, the individual molecules were used to stimulate peripheral lymphocytes isolated from *M. bovis*-infected cattle. Only $AcPIM_6$ drove significant levels of IFN-γ from PBMC (Fig. 2A). Interestingly, when whole blood taken from the same animals was stimulated overnight with the PIM molecules as previously described (25-27), no IFN-γ could be measured, and this was not due to a lack of viability as stimulation with pokeweed mitogen (PWM) generated high levels of IFN-γ (data not shown). Although no IFN-γ production was seen from the whole-blood assay, $AcPIM_6$ was able to drive significant production of IFN-γ from PBMC incubated for 5 days (Fig. 2A).

There are very few studies showing the effect of mycobacterial lipids in short-incubation, whole-blood assays. Cell-mediated immune responses to lipid antigens are more commonly assessed by enzyme-linked immunosorbent spot (ELISpot) assay, with incubation times of at least 48 h required before measurable responses become apparent (24, 28). Further, the requirement for antigen processing and presentation of specific PIMs has been demonstrated previously (20); perhaps the most likely explanation for the discrepancy between whole-blood and PBMC IFN-γ responses is that the frequencies of lipid-responsive cells are low and that an extended incubation allows for expansion of these cells. This is supported by our demonstration of strong proliferative responses induced after stimulation of PBMC with PIMs (Fig. 2B).

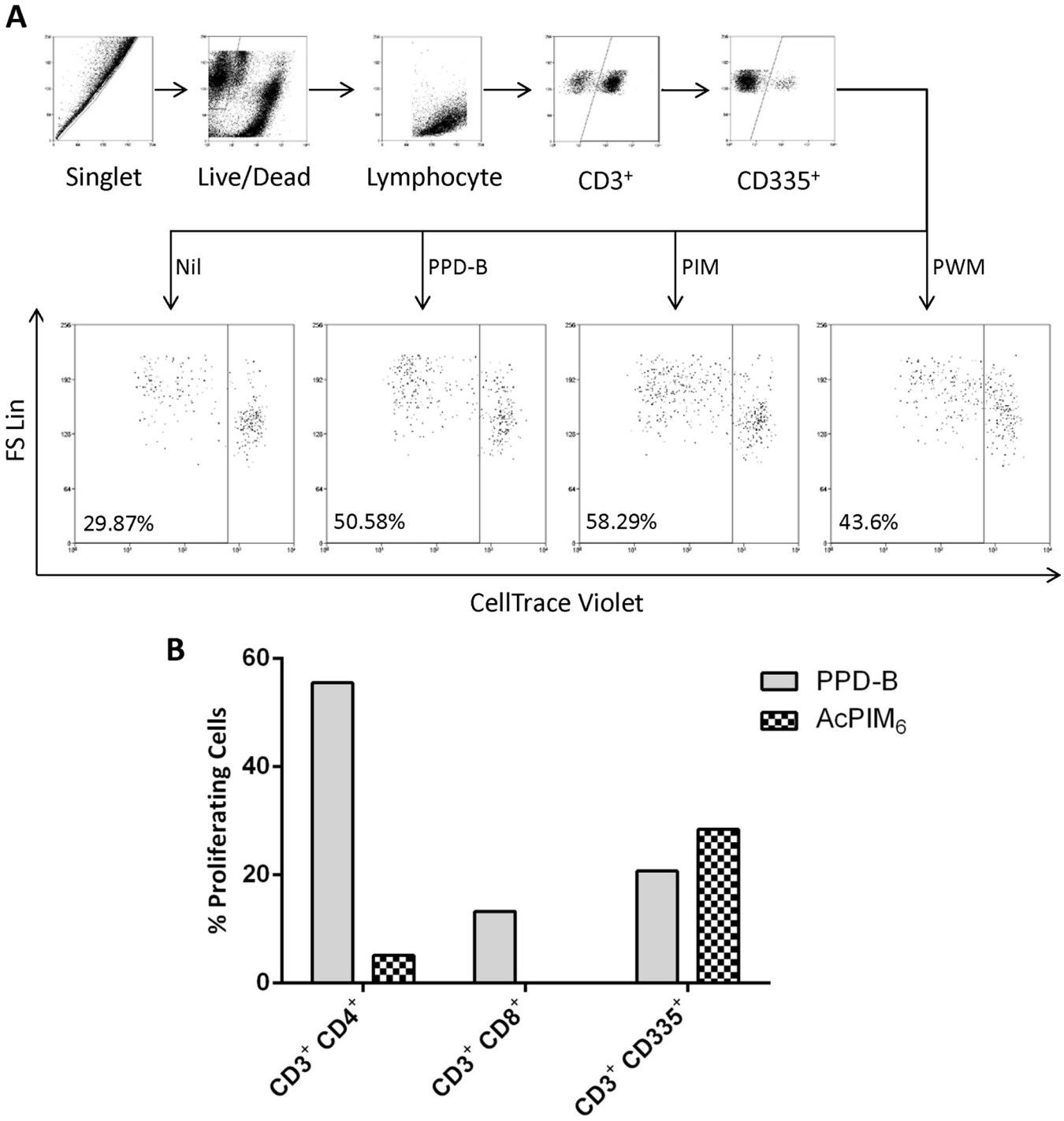

FIG 3 Assessment of proliferating cell phenotype by flow cytometry. (A) Flow cytometric gating strategy. Single, live $CD3^+$ $CD335^+$ lymphocytes were assessed for CellTrace violet labeling, and cells expressing low levels of CellTrace violet were gated for phenotyping. Numbers represent the percentages of proliferating cells in response to each antigen. FS Lin, forward scatter (linear). (B) Proliferation of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, or $CD3^+$ $CD335^+$ cells in response to either PPD-B or $AcPIM_6$. Each bar represents the percentage of cells proliferating after subtraction of the unstimulated control.

A recent study has shown that bovine NKT cells are present only at low frequencies (0.1% to 1.7%) (29).

Previous work using mice has shown that the ability of PIMs to generate cell-mediated responses is dependent on the acyl structures of the molecules. Early work performed using PIM_2 and PIM_6 demonstrated that the acyl chain was essential for NKT cell recruitment while the complexity of the mannose residues did not alter the response (23); however, it was subsequently shown that the second acyl chain of PIM_4 enhances binding to murine CD1d but that the polar mannose head was essential for antigen recognition, proliferation, and IFN-γ production (28). As well as the number and location of acyl chains, their degree of unsaturation

and *cis*, but not *trans*, stereochemistry are critical in determining antigenicity (14, 30).

The larger and more complex PIM molecules tested here (Ac_2PIM_2, Ac_2PIM_6, and $AcPIM_6$) drove significant levels of proliferation. $AcPIM_2$ also drove proliferation in 3 animals while PIM_2 generated no positive responses.

As $AcPIM_6$ was the only molecule to drive significant IFN-γ responses in our study (Fig. 2A) and one of the most potent inducers of proliferation (Fig. 2B), we decided to use $AcPIM_6$ to characterize the proliferative response. Stimulation with $AcPIM_6$ induced higher levels of proliferation in NKT cells than in $CD4^+$ or $CD8^+$ T cells (Fig. 3). However, from these data it is not possible to tell if the proliferative $CD4^+$ or $CD8^+$ cells also coexpress CD335 as our flow cytometric labeling panels do not allow the discrimination; however, this is a distinct possibility.

Although well characterized in humans and mice, the presence of NKT cells in cattle has been a controversial issue (31–34). Nevertheless, studies have shown that the bovine CD1D gene is expressed and translated *in vivo* (35), and recent work has identified a subset of cattle lymphocytes that express both T cell (CD3) and NK cell (NKp46) markers, suggesting the presence of an NKT cell population in bovine peripheral blood (29). Furthermore, bovine NKT cells have been shown to express both αβ- and γδ-TCRs, to have a broad TCR repertoire, and to have fully functional NKp46, CD16, and CD3 signaling pathways (29). Interestingly, these cells require ligation of their CD3 molecules to produce IFN-γ. While this may initially suggest that a CD3 binding component may be present in our PIM preparations, it is worth noting that we have not identified the cytokine-producing cells.

The identification of $AcPIM_6$ as a potent immunostimulatory molecule is of great interest both as a potential vaccine candidate (21), an adjuvant formulation (36), or a target for attenuation in the development of novel live vaccines (37). PIMs have also been used previously as diagnostic reagents for both tuberculosis and leprosy, although with limited success (38).

In conclusion, we present here the ability to extract and selectively purify PIMs to a high level of purity. These molecules could be used to stimulate significant IFN-γ production and drive significant proliferation in PBMC from cattle. We have also been able to identify the proliferative population and, for the first time, we have shown antigen-specific NKT activation in cattle.

ACKNOWLEDGMENTS

This study was funded by the Department for Environment, Food and Rural Affairs, United Kingdom (EMIDA-funded project Mycobactdiagnosis).

We sincerely appreciate the staff of the Animal Services Unit at AHVLA for their dedication to the welfare of test animals. We also thank Florence Dufreneix for her technical assistance and Buko Lindner (Research Centre Borstel) for ESI-mass spectrometry.

We have no competing interests.

REFERENCES

1. **Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D.** 1995. Structural definition of acylated phosphatidylinositol mannosides from *Mycobacterium tuberculosis*: definition of a common anchor for lipomannan and lipoarabinomannan. Glycobiology **5:**117–127. http://dx.doi.org/10.1093/glycob/5.1.117.
2. **Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, Matsumoto R, Rosat JP, Modlin RL, Porcelli SA.** 1996. CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. J Immunol **157:**2795–2803.
3. **Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB.** 1994. Recognition of a lipid antigen by CD1-restricted ab^+ T cells. Nature **372:**691–694. http://dx.doi.org/10.1038/372691a0.
4. **Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ.** 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. Science **269:**227–230. http://dx.doi.org/10.1126/science.7542404.
5. **Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M.** 1997. CD1d-restricted and TCR-mediated activation of Va14 NKT cells by glycosylceramides. Science **278:**1626–1629. http://dx.doi.org/10.1126/science.278.5343.1626.
6. **Spada FM, Koezuka Y, Porcelli SA.** 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med **188:**1529–1534. http://dx.doi.org/10.1084/jem.188.8.1529.
7. **Behar SM, Podrebarac TA, Roy CJ, Wang CR, Brenner MB.** 1999. Diverse TCRs recognize murine CD1. J Immunol **162:**161–167.
8. **Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D.** 1995. CD1-restricted $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. J Exp Med **182:**993–1004. http://dx.doi.org/10.1084/jem.182.4.993.
9. **Chiu YH, Jayawardena J, Weiss A, Lee D, Park SH, Dautry-Varsat A, Bendelac A.** 1999. Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. J Exp Med **189:**103–110. http://dx.doi.org/10.1084/jem.189.1.103.
10. **Yoshimoto T, Bendelac A, Watson C, Hu-Li J, Paul WE.** 1995. Role of $NK1.1^+$ T cells in a Th2 response and in immunoglobulin E production. Science **270:**1845–1847. http://dx.doi.org/10.1126/science.270.5243.1845.
11. **Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, Koezuka Y, Porcelli SA, Cardell S, Brenner MB, Behar SM.** 2000. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity **12:**211–221. http://dx.doi.org/10.1016/S1074-7613(00)80174-0.
12. **Li S, Choi HJ, Felio K, Wang CR.** 2011. Autoreactive CD1b-restricted T cells: a new innate-like T-cell population that contributes to immunity against infection. Blood **118:**3870–3878. http://dx.doi.org/10.1182/blood-2011-03-341941.
13. **Kinjo Y, Kronenberg M.** 2005. Va14 i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. J Clin Immunol **25:**522–533. http://dx.doi.org/10.1007/s10875-005-8064-5.
14. **Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, Zajonc DM, Ben-Menachem G, Ainge GD, Painter GF, Khurana A, Hoebe K, Behar SM, Beutler B, Wilson IA, Tsuji M, Sellati TJ, Wong CH, Kronenberg M.** 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat Immunol **7:**978–986. http://dx.doi.org/10.1038/ni1380.
15. **Skold M, Behar SM.** 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect Immun **71:**5447–5455. http://dx.doi.org/10.1128/IAI.71.10.5447-5455.2003.
16. **Bligh EG, Dyer WJ.** 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:**911–917. http://dx.doi.org/10.1139/o59-099.
17. **Ainge GD, Parlane NA, Denis M, Dyer BS, Harer A, Hayman CM, Larsen DS, Painter GF.** 2007. Phosphatidylinositol mannoside ether analogues: syntheses and interleukin-12-inducing properties. J Org Chem **72:**5291–5296. http://dx.doi.org/10.1021/jo070639m.
18. **Doz E, Rose S, Court N, Front Vasseur SV, Charron S, Gilleron M, Puzo G, Fremaux I, Delneste Y, Erard F, Ryffel B, Martin OR, Quesniaux VF.** 2009. Mycobacterial phosphatidylinositol mannosides negatively regulate host Toll-like receptor 4, MyD88-dependent proinflammatory cytokines, and TRIF-dependent co-stimulatory molecule expression. J Biol Chem **284:**23187–23196. http://dx.doi.org/10.1074/jbc.M109.037846.
19. **Court N, Rose S, Bourigault M-L, Front S, Martin OR, Dowling JK, Kenny EF, O'Neill L, Erard F, Quesniaux VF.** 2011. Mycobacterial PIMs inhibit host inflammatory responses through CD14-dependent and CD14-independent mechanisms. PLoS One **6:**e24631. http://dx.doi.org/10.1371/journal.pone.0024631.
20. **Cala-De Paepe D, Layre E, Giacometti G, Garcia-Alles LF, Mori L, Hanau D, de Libero G, de la Salle H, Puzo G, Gilleron M.** 2012. Deciphering the role of CD1e protein in mycobacterial phosphatidyl-*myo*-inositol mannosides (PIM) processing for presentation by CD1b to T lymphocytes. J Biol Chem **287:**31494–31502. http://dx.doi.org/10.1074/jbc.M112.386300.
21. **Parlane NA, Compton BJ, Hayman CM, Painter GF, Basaraba RJ,**

Heiser A, Buddle BM. 2012. Phosphatidylinositol di-mannoside and derivates modulate the immune response to and efficacy of a tuberculosis protein vaccine against *Mycobacterium bovis* infection. Vaccine **30:**580–588. http://dx.doi.org/10.1016/j.vaccine.2011.11.055.

22. **Gilleron M, Quesniaux VF, Puzo G.** 2003. Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guerin and *Mycobacterium tuberculosis* H37Rv and its implication in Toll-like receptor response. J Biol Chem **278:**29880–29889. http://dx.doi.org/10.1074/jbc.M303446200.
23. **Gilleron M, Ronet C, Mempel M, Monsarrat B, Gachelin G, Puzo G.** 2001. Acylation state of the phosphatidylinositol mannosides from *Mycobacterium bovis* bacillus Calmette Guerin and ability to induce granuloma and recruit natural killer T cells. J Biol Chem **276:**34896–34904. http://dx.doi.org/10.1074/jbc.M103908200.
24. **Sprott GD, Dicaire CJ, Gurnani K, Sad S, Krishnan L.** 2004. Activation of dendritic cells by liposomes prepared from phosphatidylinositol mannosides from *Mycobacterium bovis* bacillus Calmette-Guerin and adjuvant activity *in vivo*. Infect Immun **72:**5235–5246. http://dx.doi.org/10.1128/IAI.72.9.5235-5246.2004.
25. **Cockle PJ, Gordon SV, Lalvani A, Buddle BM, Hewinson RG, Vordermeier HM.** 2002. Identification of novel *mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect Immun **70:**6996–7003. http://dx.doi.org/10.1128/IAI.70.12.6996-7003.2002.
26. **Ewer K, Cockle PJ, Gordon SV, Mansoor H, Govaerts M, Walravens K, Marche S, Hewinson RG, Vordermeier HM.** 2006. Antigen mining with iterative genome screens identifies novel diagnostics for the *mycobacterium tuberculosis* complex. Clin Vaccine Immunol **13:**90–97. http://dx.doi.org/10.1128/CVI.13.1.90-97.2006.
27. **Jones GJ, Gordon SV, Hewinson RG, Vordermeier HM.** 2010. Screening of predicted secreted antigens from *Mycobacterium bovis* reveals the immunodominance of the ESAT-6 protein family. Infect Immun **78:**1326–1332. http://dx.doi.org/10.1128/IAI.01246-09.
28. **Fischer K, Scotet E, Niemeyer M, Koebernick H, Zerrahn J, Maillet S, Hurwitz R, Kursar M, Bonneville M, Kaufmann SH, Schaible UE.** 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. Proc Natl Acad Sci U S A **101:**10685–10690. http://dx.doi.org/10.1073/pnas.0403787101.
29. **Connelley TK, Longhi C, Burrells A, Degnan K, Hope J, Allan AJ, Hammond JA, Storset AK, Morrison WI.** 2014. NKp46$^+$ CD3$^+$ cells: a novel nonconventional T cell subset in cattle exhibiting both NK cell and T cell features. J Immunol **192:**3868–3880. http://dx.doi.org/10.4049/jimmunol.1302464.
30. **Rauch J, Gumperz J, Robinson C, Skold M, Roy C, Young DC, Lafleur M, Moody DB, Brenner MB, Costello CE, Behar SM.** 2003. Structural features of the acyl chain determine self-phospholipid antigen recognition by a CD1d-restricted invariant NKT (iNKT) cell. J Biol Chem **278:**47508–47515. http://dx.doi.org/10.1074/jbc.M308089200.
31. **Chen YH, Chiu NM, Mandal M, Wang N, Wang CR.** 1997. Impaired NK1$^+$ T cell development and early IL-4 production in CD1-deficient mice. Immunity **6:**459–467. http://dx.doi.org/10.1016/S1074-7613(00)80289-7.
32. **Gapin L, Matsuda JL, Surh CD, Kronenberg M.** 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. Nat Immunol **2:**971–978. http://dx.doi.org/10.1038/ni710.
33. **Looringh van Beeck FA, Reinink P, Hermsen R, Zajonc DM, Laven MJ, Fun A, Troskie M, Schoemaker NJ, Morar D, Lenstra JA, Vervelde L, Rutten VP, van Eden W, Van Rhijn I.** 2009. Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants. Mol Immunol **46:**1424–1431. http://dx.doi.org/10.1016/j.molimm.2008.12.009.
34. **Van Rhijn I, Koets AP, Im JS, Piebes D, Reddington F, Besra GS, Porcelli SA, van Eden W, Rutten VP.** 2006. The bovine CD1 family contains group 1 CD1 proteins, but no functional CD1d. J Immunol **176:**4888–4893. http://dx.doi.org/10.4049/jimmunol.176.8.4888.
35. **Nguyen TK, Koets AP, Vordermeier M, Jervis PJ, Cox LR, Graham SP, Santema WJ, Moody DB, van Calenbergh S, Zajonc DM, Besra GS, Van Rhijn I.** 2013. The bovine CD1D gene has an unusual gene structure and is expressed but cannot present a-galactosylceramide with a C26 fatty acid. Int Immunol **25:**91–98. http://dx.doi.org/10.1093/intimm/dxs092.
36. **Wedlock DN, Denis M, Painter GF, Ainge GD, Vordermeier HM, Hewinson RG, Buddle BM.** 2008. Enhanced protection against bovine tuberculosis after coadministration of *Mycobacterium bovis* BCG with a Mycobacterial protein vaccine-adjuvant combination but not after coadministration of adjuvant alone. Clin Vaccine Immunol **15:**765–772. http://dx.doi.org/10.1128/CVI.00034-08.
37. **Mahon RN, Rojas RE, Fulton SA, Franko JL, Harding CV, Boom WH.** 2009. *Mycobacterium tuberculosis* cell wall glycolipids directly inhibit CD4$^+$ T-cell activation by interfering with proximal T-cell-receptor signaling. Infect Immun **77:**4574–4583. http://dx.doi.org/10.1128/IAI.00222-09.
38. **Mullins RJ, Fournie JJ, Moloney B, Baumgart K, Jones P, Brown P, Basten A.** 1992. Serological response to purified mycobacterial phosphatidylinositol mannoside in healthy controls and in patients with tuberculosis and leprosy. Int J Lepr Other Mycobact Dis **60:**353–367.